Conversion of Tannic Acid to Gallic Acid

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VIJAY KUMAR ARORA

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CERTIFICATE

It is certified that this work has been carried out under my supervision and that this has not been submitted elsewhere for a degree.

P.S. Ramachandran
Assistant Professor
Department of Chemical Engineering
Indian Institute of Technology
Kanpur

10th August 1970

373

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Author

CONTENTS

* * *

LIST OF TABLES

* * *

LIST OF FIGURES

* * *

ABSTRACT

Studies have been made to produce gallic acid by fermentation using *Aspergillus niger* with tannic acid as substrate. Preliminary investigations were made to get the favourable values of temperature and pH of fermentation. A temperature of 30°C and a pH of 5.5 were found suitable for the conversion when the substrate concentration was 2% tannic acid.

The chemical changes during fermentation of tannic acid to gallic acid by *A. niger* for three different initial tannic acid concentrations of 0.5, 2.0 and 5.0% are presented. 2% concentration is found to yield the maximum conversion of tannic acid to gallic acid at a period of about 28 hours. However, the growth is maximum at 48 - 52 hours of incubation at the conditions.

For the kinetics of growth of *A. niger* on substrate containing tannic acid initially, it is found that the growth follows cube-root law more closely than the exponential law.

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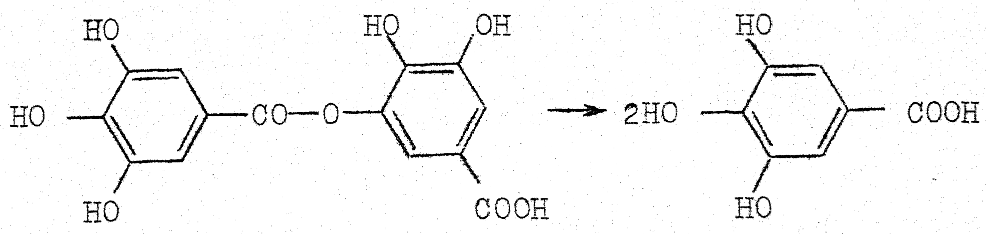
CHAPTER I

INTRODUCTION

Gallic acid (3,4,5-trihydroxybenzoic acid) finds important uses in the tanning, printing, engraving and pharmaceutical industries.

The acid occurs naturally in ester or glycoside combination in plant tannins. It can be prepared by chemical or enzymic hydrolysis of tannins. Tannins are supposed to be a mixture of derivatives of polyhydroxy-benzoic acids and occur in substances of vegetable origin like sumac, gallnuts, myrobalans, root bark, chinese tea, etc. Tannin is referred to as tannic acid, or gallo-tannin or digallic acid.

In chemical hydrolysis, the tannins (after extracting from tannin yielding materials) are hydrolysed by boiling with water in the presence of some acid or alkali, which act as a catalyst. The hydrolysis reaction of tannic acid to gallic acid is believed to be (1):



Digallic acid   Gallic acid

In enzymic hydrolysis, hydrolysis of tannins is brought about by the action of certain molds (fermenting culture) e.g. *Aspergillus*, *Penicillium*. Out of these, *Aspergillus niger* appears to be more suitable for the hydrolysis. In this method, aqueous tannin extract from tannin yielding materials or tannic acid along with other necessary nutrients are inoculated with selected strains of *Aspergillus niger*. The solution is agitated and aerated until hydrolysis of tannins is complete. The product is crystallized from the fermented liquor.

The conversion of tannins to gallic acid is brought about by means of the enzyme "tannase". The mold develops at the expense of utilizable nutrients in the extract, producing extracellular enzyme tannase, which hydrolytically produces gallic acid.

In the present study, submerged gallic acid fermentation was carried out, with gallic acid being produced on the growth medium. The effect of temperature and pH of fermenting medium on the gallic acid yield were investigated

in the preliminary studies for a substrate concentration of 2% tannic acid and fermentation time of 24 hours. Also, the chemical changes during the course of fermentation were studied for substrate concentrations of 0.5, 2.0 and 5.0 % tannic acid at optimal condition of temperature and pH as found out in the preliminary studies.

In order to establish the growth kinetics of Aspergillus niger on substrates containing tannic acid initially, attempts were made to see whether the growth data follow cube root law or exponential law. Least square method was used to determine the correlation coefficient, sum of the squares of errors, the slope and 95 percent confidence limits on the slope for both the cases.

It is hoped that the present work, though not exhaustive, would help in the understanding of the problem of conversion of tannins to gallic acid by Aspergillus niger and throws some light on the growth kinetics of the fungus.

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CHAPTER II

LITERATURE REVIEW

GALLIC ACID was first discovered by Schelle (2) in 1787. He noticed that a cold water infusion of gallnuts containing tannins had deposited a sediment which also gave a black precipitate with iron sulphate just like tannic acid, but was not identical with the original tannin. It had a sour taste, rather than an astringent taste which is characteristic of tannic acid.

In one of the oldest methods used for the production of gallic acid by fermentation, the substances containing tannins were piled up in heaps and moistened with water. Molds developed throughout the heap, which was stirred occasionally and maintained at a temperature of approximately 30°C. After a fermentation period of about a month, the gallic acid was leached from the heap.

Van Tiegham (3) reported that there are two groups of molds, namely, *Aspergillus niger* and *Penicillium glaucum* which would produce gallic acid by their growth on a tannin liquor. He also found that air was essential for the fermentation process. Later, Fernback (4) and Pottevin (5) independently reported that the enzyme 'tannase' was responsible for the hydrolysis reaction. They observed that tannase was produced by the growth of *Aspergillus niger* on tannin solutions containing suitable nutrients. Their results prove conclusively that the hydrolysis of tannin to gallic acid was an extracellular reaction.

Calmette (6) in a patent claimed that a specific organism, which he named as *Aspergillus gallomyces* is found in small quantities in gallnuts. By suitable methods, he prepared a pure culture of this organism which was then inoculated into a sterile tank containing tannins. He kept the organism under submerged conditions, both by means of a mechanical agitator and by the introduction of a large quantity of sterile air. Fermentation was terminated when the tannin disappeared completely and the resulting gallic acid was recovered in the usual manner.

Knudson (7) reported that the tannase content of Aspergillus niger could be increased by partially replacing sucrose in Czapek's solution by tannic acid. Czapek's solution consists of sucrose, sodium nitrate, potassium phosphate, potassium chloride, magnesium sulphate and ferrous sulphate. Nicholson et.al. (8) found that in addition to tannase, another enzyme known as "Pyrogallase" is produced in the reaction mixture which destroys gallic acid.

Steinberg (9) found that although iron, zinc, copper, manganese were necessary for the growth of Aspergillus niger, molybdenum and gallium were essential for the growth. He also reported that nitrates, ammonium salts and nitrohydroxylamic acid salts were better sources of inorganic nitrogen for the growth than nitrites, hyponitrites, hydrazines, azides and nitrous oxide.

Metabolism of gallic acid by Aspergillus niger was studied by Akira Watanabe (10). He found that strains of Aspergillus and Penicillium can assimilate gallic acid as a sole carbon-source. Gallic acid was completely consumed in 90 hours when a medium containing 0.2% acid was used for the study. When media containing 3% glucose and 0.5% gallic

acid was used, he observed that glucose was first utilized for the growth, later followed by the gallic acid consumption.

Very little work has been done for the isolation of the enzyme "tannase", which can be directly used as a catalyst for the conversion of tannic acid to gallic acid. Nishira, H. and N. Mugibayashi (11) report that the tannase can be prepared by salting out with ammonium sulphate from the mycelium extract of 4% tannic acid, containing bran culture of Penicillium, sp. No. 70B'. The tannase was also prepared from the culture liquid and from the mycelium extract of Penicillium sp. No. 80B' which was obtained by shaking culture with Czapek-Dox solution containing 0.5% tannic acid as the secondary medium.

Recently Hideaki Yamada, et.al. (12) claimed that tannase was found in the mycelium of Aspergillus flavus grown on the medium containing tannic acid as a sole carbon source. Tannase was purified from the mycelium extract by a procedure involving ammonium sulphate and tannic acid precipitation, DEAE-cellulose column chromatography, sephadex G-200 gel filtration, finally followed by acetone fractionation.

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CHAPTER III

EXPERIMENTAL

All the chemicals used in this work were of commercial grade. Pure gallic acid* was used as a standard. Tannic acid** that was used contained 10 - 12% gallic acid originally.

Three different strains of Aspergillus niger were obtained from Central Drug Research Institute, Lucknow, Central Leather Research Institute, Madras, and National Chemical Laboratory, Poona. The strain from Central Leather Research Institute was found to be unadaptable to tannic acid media whereas both the growth of mycelium and conversion of tannic acid to gallic acid were low in the case of culture from National Chemical Laboratory. The culture obtained from Central Drug Research Institute was used throughout the present study, since it was found most suitable for the present studies.

Preservation of Culture:

The strain was maintained on Czapek's - Dox medium containing 1.5 percent bacteriological agar. The composition

*Pure gallic acid, made in Germany of Chemisch Rein, Krist., DAB.6.,Hom, A.B.

**Tannic acid B.P. from W.J. Bush & Co. Limited, London

of the medium based on 1000 ml. solution was as follows:

Sucrose	30.0 g.
Sodium Nitrate($NaNO_3$)	2.0 g.
Patassium dihydrogen phosphate (KH_2PO_4)	1.0 g.
Magnesium Sulphate ($Mg\ SO_4 \cdot 7H_2O$)	0.5 g.
Potassium Chloride (KCl)	0.5 g.

Preparation of Inoculum:

A medium which will be referred to as 'Medium A' was prepared with the following composition:

Medium A

Tannic acid	20.0 g.
Sodium nitrate ($NaNO_3$)	2.0 g.
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g.
Potassium chloride (KCl)	0.5 g.
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.5 g.
Sodium tauroglycolate	0.5 g.
Water to	1000 ml.

Sodium tauroglycolate was used as a surface-active agent in 0.05% concentration in order to obtain individual colonies of homogeneous composition in liquid culture as suggested by Dorn, G (13). It is known that surface-active

agents restrict the radial growth of the fungus without affecting their viability or normal differentiation.

150 ml. of 'Medium A' was taken in a 500 ml. conical flask. It was sterilized at 5 psig for 20 minutes and inoculated with spores of <u>Aspergillus</u> <u>niger</u>. It was then incubated at a temperature of 28°C for 24 hours. 5 mls. of this (dry weight approximately 20 mg.) was used as inoculum for all the shake-flask batch culture runs. The procedure for these runs was as given below:

<u>Procedure</u>:

50 mls. of nutrient media was taken in 500 mls conical flask. Twenty flasks were used for a single run. The media was sterilized at 5 psig for 20 minutes and then cooled. 5 mls of the inoculum which was prepared as explained before, was added asceptically to each flask to start the fermentation. The flasks were then placed in a shaker in a constant temperature room. The shaker speed was adjusted such that there was sufficient agitation and at the same time it did not cause any wetting of the cotton that was used as a plug. The temperature of the constant temperature room could be set at any desired value between 20°C and 40°C.

For these studies, sucrose in Czapek-Dox medium was replaced by tannic acid. The quantities of the other

ingredients were kept at the same values as indicated earlier. Preliminary investigations using a media containing 2% tannic acid and fermentation time of 24 hours indicated that a pH of about 5.5 and temperature about 30°C were the most favourable conditions for conversion of tannin to gallic acid. The results of these investigations are shown in figures 1 and 2 later. Therefore, all the experiments were conducted at these conditions. The pH was maintained constant by using 0.05 M acetate buffer.

In order to study the effect of the growth limiting substrate on the growth of *Aspergillus niger*, and the optimum yield of gallic acid, the tannic acid concentration in the medium was varied, whereas the amounts of other components were kept the same at the values indicated earlier The runs were carried out as explained before. The chemical changes during fermentation were investigated for 0.5, 2.0 and 5.0 % tannic acid substrate concentrations.

At various times, the flasks were removed from the shaker for analysis purposes. The sample contained in the flask was filtered, and mycelium mat was washed thoroughly with distilled water. The mat was used to determine the dry weight of mycelium. The filtrate was collected and analyzed for tannic acid and gallic acid concentrations.

The procedure for determining tannic acid and gallic acid concentrations in the solution, and the dry weight of the mycelium is given in Appendix A. At each experimental condition, duplicate runs were taken in order to check for their reproducibility.

* * *

CHAPTER IV

RESULTS AND DISCUSSION

The results of the shake-flask batch culture studies for the mold growth as well as for the hydrolysis reaction are given and discussed below.

Table I summarizes the effect of pH on the yield of gallic acid when the other conditions were kept constant at the values indicated. The same results are shown in Fig. 1. pH was varied from 4.0 to 7.0. The alkaline range of pH was not studied since the fungus growth is known to be poor in alkaline medium. It is found from Fig. 1 that a pH of about 5.5 is optimum for the specified set of conditions.

The effect of temperature of fermentation on the yield of the product is shown in Fig. 2. The results are tabulated in Table II. Since a pH of 5.5 was found most suitable for conversion, the pH was maintained at this value to study the effect of temperature. The temperature was varied from 25° to 40°C. It is seen that the optimum temperature is about 30°C for the set of conditions specified on the figure.

TABLE I

EFFECT OF pH ON CONVERSION

Substrate Concentration = 2.00% Tannic Acid

Fermentation Time = 24 hours

Temperature = 28°C

pH	Gallic Acid mg/ml
4.0	5.8
4.5	8.7
5.0	9.8
5.5	10.6
6.0	10.0
6.5	9.0
7.0	8.6

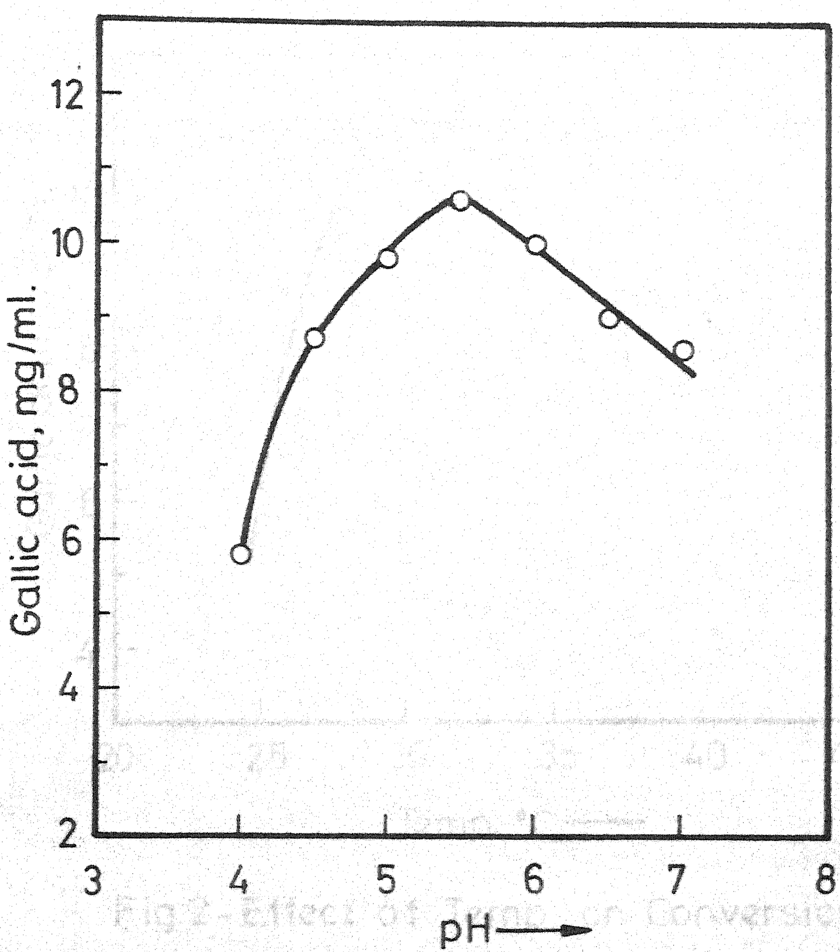

Fig. 1 - Effect of pH on conversion.

Conditions: Substrate Conc. 2% tannic acid.

Temp. = 28°C

Time of fermentation = 24 hrs.

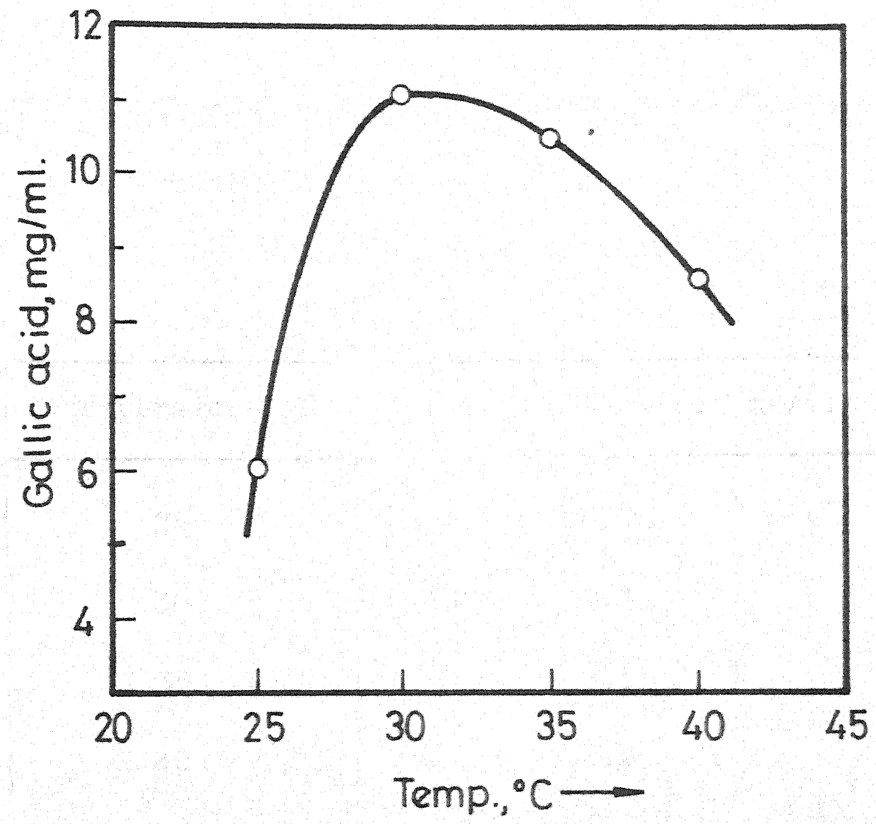

Fig.2 - Effect of Temp. on Conversion.

Conditions : Substrate Conc. 2% tannic acid.

pH = 5.5

Time of fermentation = 24hrs.

TABLE II

EFFECT OF TEMPERATURE ON CONVERSION

Substrate Concentration = 2.00% Tannic Acid

Fermentation Time = 24 hours

pH = 5.5 (0.05 M Citrate Buffer)

Temperature °C	Gallic Acid mg/ml
25	6.0
30	11.1
35	10.5
40	8.6

The results of the three sets of batch submerged culture, with initial tannic acid concentration of 0.5, 2.0 and 5.0% (weight basis) are presented in Tables III, IV and V and are shown graphically in Figures 3, 4, and 5 respectively. The graphs show the amount of gallic acid formed, tannic acid left in the culture broth and the dry weight of mycelium mat at various times during a run. At each set of conditions, two runs were made to check for the reproducibility. The data of both the runs are shown in the Figures. The figures indicate that the runs are reproducible reasonably well within experimental accuracy. The yield coefficient is determined for each set at the time of maximum product formation. Yield coefficient is defined in the present study, as the ratio of amount of gallic acid formed to the amount of tannic acid consumed. These values are tabulated in Table VI.

Figure 3 indicates that there is no product formation for the first 8 hours. The maximum product formation occurs after 24 hours of incubation. From this point, the concentration of gallic acid in the fermentation mixture starts decreasing at a faster rate. The rate of utilization of tannic acid is very fast for the first 24 hours and then the rate decreases slowly. After about 52 hours of incubation, almost all the tannic acid is consumed. Fig. 3

TABLE III

CHEMICAL CHANGES DURING FERMENTATION

C_{Si} = 0.5% Tannic Acid

Temp. = 30°C

pH = 5.5

Time Hours	Mycelial Weight mg/50 ml.		Gallic Acid mg/ml.	Tannic Acid + Gallic Acid mg/ml.	Tannic Acid equivalent gallic acid mg./ml.	Tannic acid mg/ml.
0	29.5	31.8	2.08	5.00	2.92	4.17
8	32.6	32.2	2.10	4.00	1.90	2.72
18	43.2	46.7	2.70	3.55	0.85	1.21
24	57.9	61.0	2.80	3.20	0.40	0.57
28	71.3	75.1	2.65	3.05	0.40	0.57
32	80.5	63.9	2.34	2.95	0.24	0.34
44	110.0	95.7	1.44	1.54	0.10	0.14
52	121.9	125.5	0.80	0.87	0.07	0.10
70	117.0	117.2	0.10	0.12	0.02	0.03

TABLE IV

CHEMICAL CHANGES DURING FERMENTATION

C_{Si} = 2.0% Tannic Acid

Temp. = 30°C

pH = 5.5

Time Hours	Mycelial Weight mg/50 ml.		Gallic Acid mg./ml.		Tannic Acid + Gallic Acid mg/ml.		Tannic Acid equivalent gallic acid mg/ml.		Tannic acid mg/ml.	
0	21.7	18.8	7.0	7.1	16.5	16.5	9.5	9.4	13.6	13.5
7	25.6	32.5	7.1	7.1	14.4	14.5	7.3	7.4	10.5	10.6
12	35.3	38.6	7.3	7.5	13.1	13.1	5.8	5.6	8.3	8.0
22	49.4	55.3	10.2	10.5	13.4	13.5	3.2	3.0	4.5	4.3
28	66.0	78.7	11.4	11.6	14.2	14.0	2.8	2.4	4.0	3.5
36	85.7	93.4	11.0	11.6	13.3	14.0	2.3	2.4	3.3	3.4
46	110.0	112.9	11.0	11.0	12.7	12.8	1.7	1.8	2.4	2.6
55	146.0	148.2	10.2	10.2	11.6	11.6	1.4	2.0	2.0	2.0
70	144.3	145.0	9.4	9.4	10.6	10.6	1.2	1.2	1.7	1.7

TABLE V

CHEMICAL CHANGES DURING FERMENTATION

C_{Si} = 5.0% Tannic Acid

Temp. = 30°C

pH = 5.5

Time Hours	Mycelial Weight mg/50 ml.		Gallic Acid mg/ml.		Tannic Acid + Gallic Acid mg/ml.		Tannic Acid equivalent gallic acid mg./ml.		Tannic Acid mg/ml.	
0	19.5	22.6	13.6	13.6	38.8	38.8	25.2	25.2	36.0	36.0
12	21.0	16.0	14.0	13.4	30.1	30.0	16.1	16.6	23.0	23.7
20	23.3	28.2	14.6	14.5	28.6	30.1	14.0	15.6	20.0	22.3
28	41.8	38.0	16.0	17.1	29.9	29.9	13.9	12.8	19.9	18.3
36	53.9	50.0	20.0	20.0	32.3	32.5	12.3	12.5	17.6	17.9
45	75.1	68.9	19.2	19.4	30.6	30.6	11.4	11.2	16.3	16.0
49	77.8	76.9	18.8	19.2	30.2	30.2	11.4	11.0	16.3	15.8
59	93.2	85.8	19.2	19.1	30.2	30.3	11.0	11.2	15.8	16.0
77	89.7	87.0	18.8	19.0	29.2	29.4	10.8	10.4	15.4	14.9

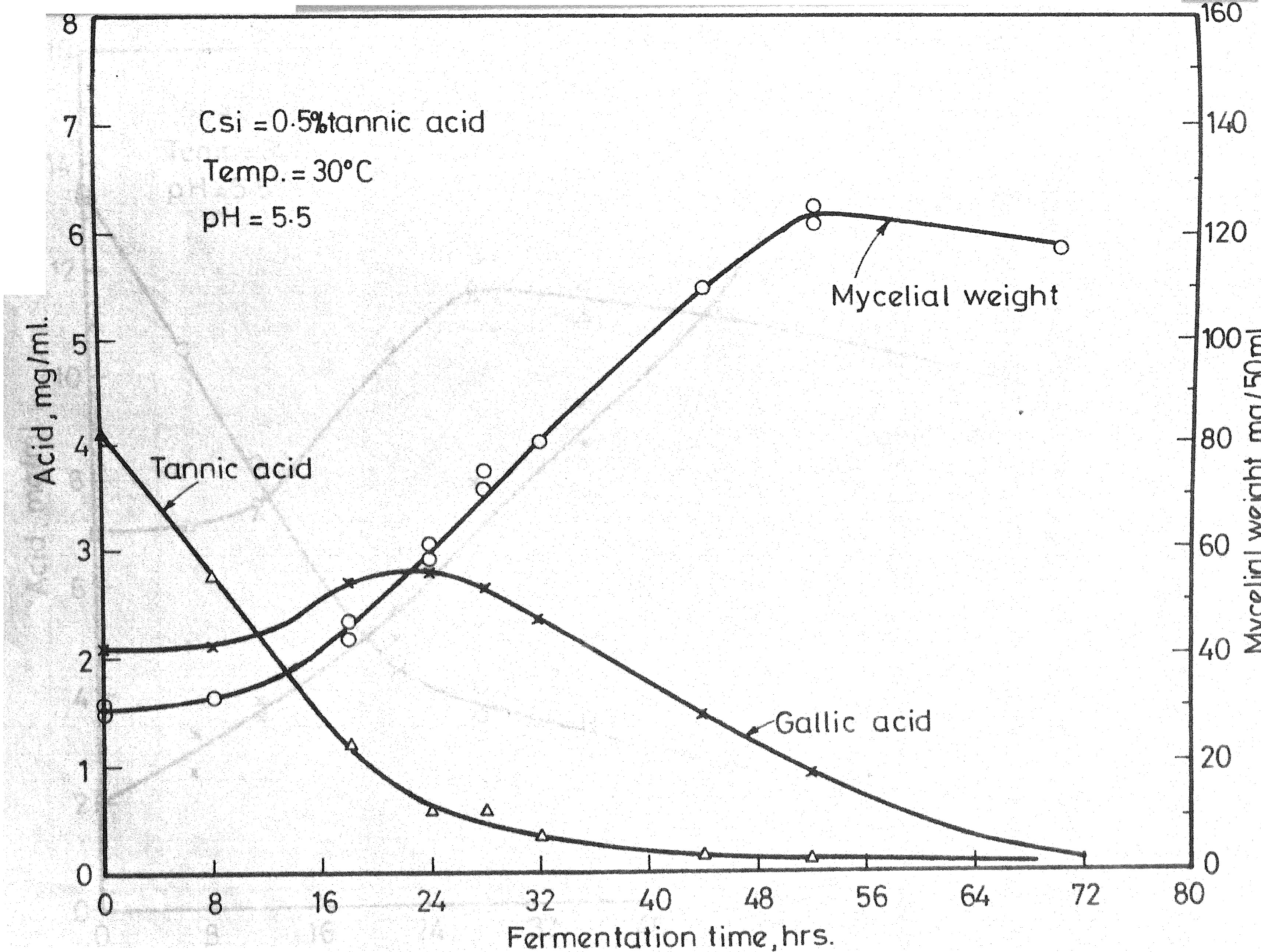

Fig. 3 - Chemical changes during fermentation.

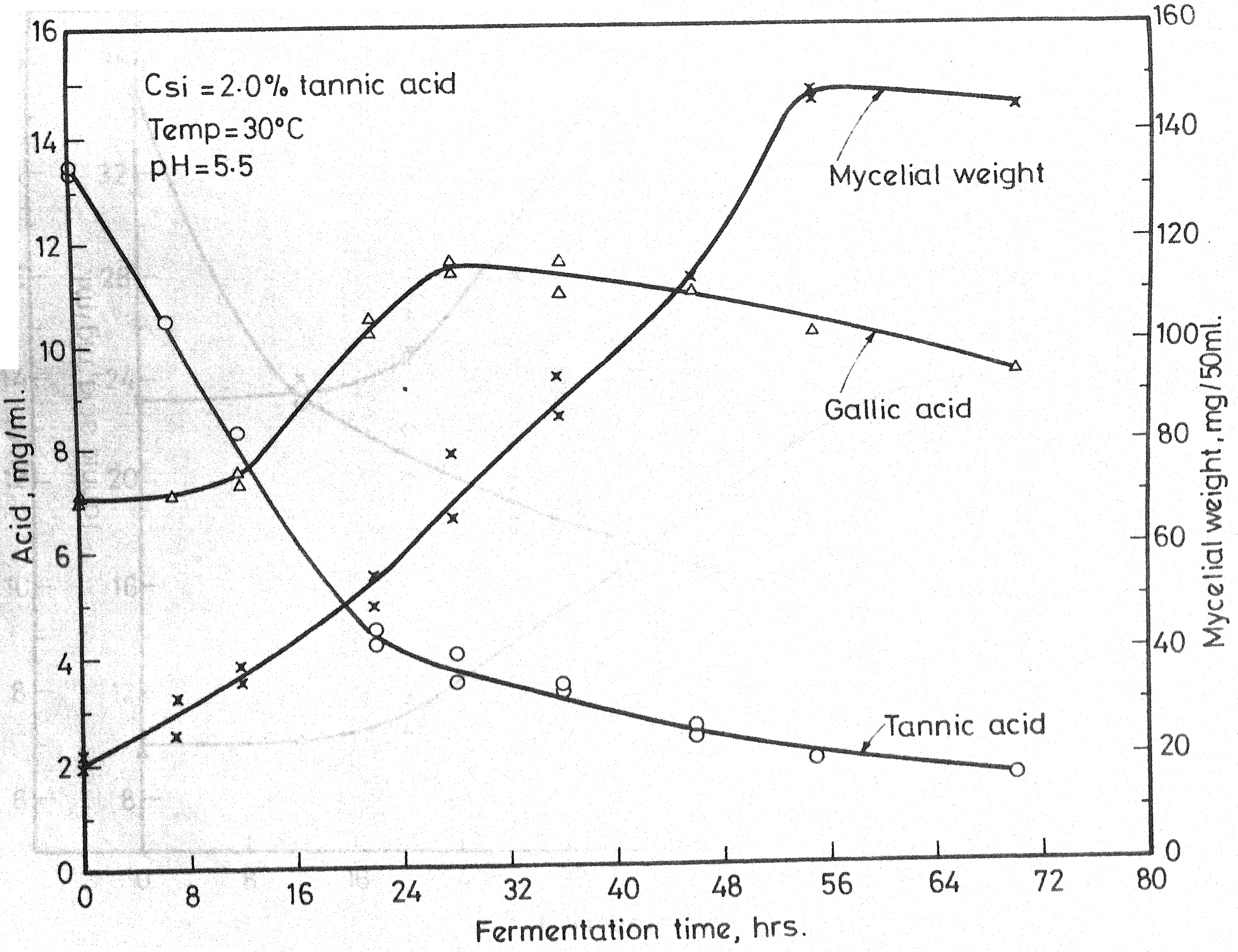

Fig. 4 - Chemical changes during fermentation.

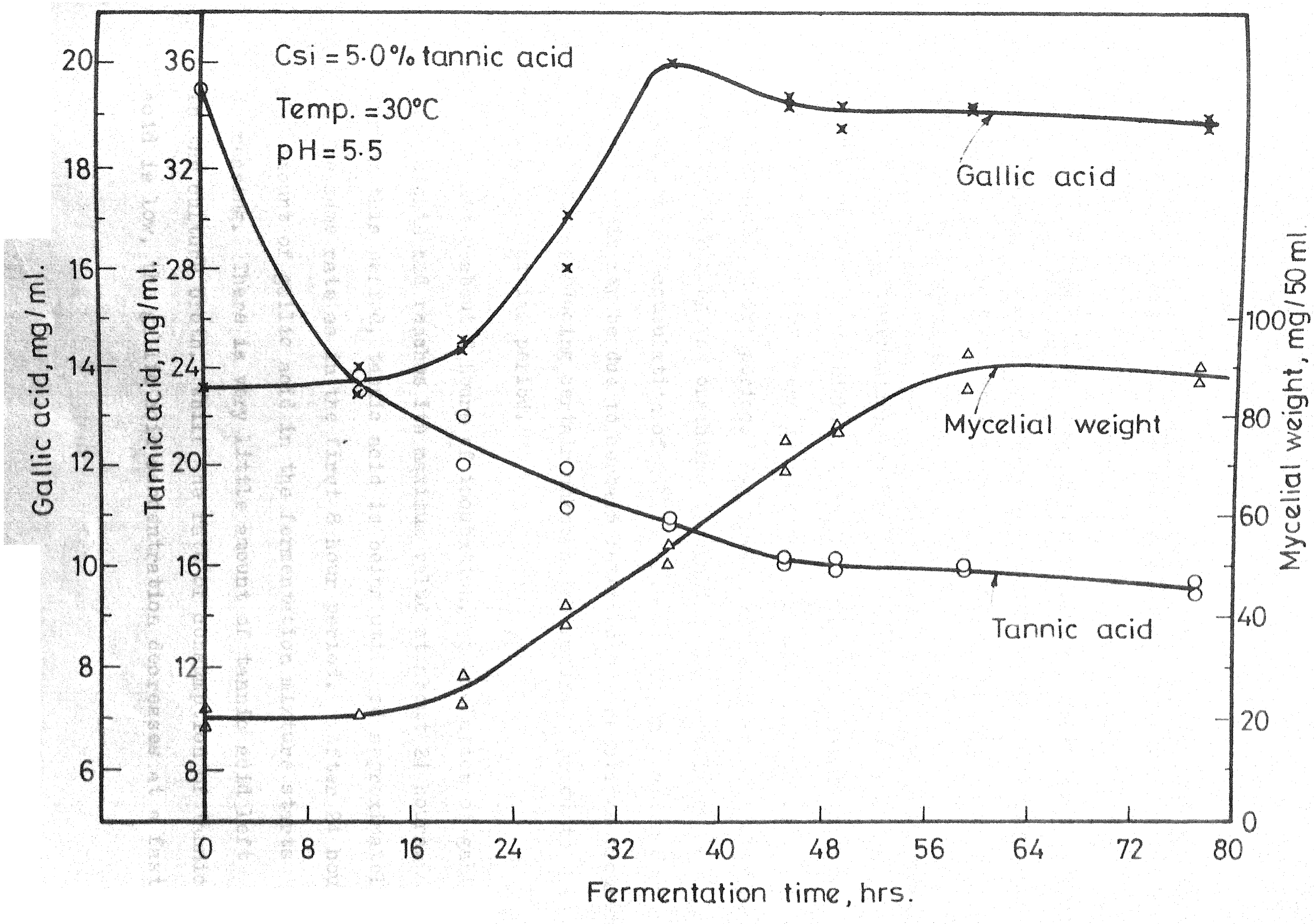

Fig.5 - Chemical changes during fermentation.

also shows that there is a lag phase of about 8 hours for the growth of organisms. This time may be required for the organisms to adapt themselves to the substrate. The growth starts after this period and increases and reaches the maximum value at 52 hours and then there is a slight decline in the dry weight of mycelium.

As the fermentation starts, the rate of utilization of tannic acid is quite high. There is a slight increase in the mycelial weight but there is no formation of gallic acid during the initial 8 hours period. Lag phase in the growth is due to the time taken by the organisms to acclimatise to the new conditions. Accumulation of gallic acid starts only after 8 hours. This may be due to certain intermediate reactions occuring in solution,producing certain unknown intermediate products during the initial period.

After about 8 hours of incubation, accumulation of gallic acid starts and reaches its maximum value at about 24 hours. During this period, tannic acid is being utilized approximately at the same rate as in the first 8 hour period. After 24 hours, the amount of gallic acid in the fermentation mixture starts decreasing. There is very little amount of tannic acid left in the culture broth. While the rate of consumption of tannic acid is low, the gallic acid concentration decreases at a faster

rate and at the same time, there is a continuous increase in the mycelial weight. From this, it can be inferred that the organisms utilize gallic acid also as a substrate for their growth. This finding is in confirmation with that of Akira Watanabe (10), that strains of Aspergillus niger and Penicillium assimilate gallic acid as a sole-carbon source. The yield coefficient for this case was found to be 0.20.

At an incubation period of 52 hours, the mycelial weight is maximum. After this period, when almost all the tannic acid is exhausted, and there is very little gallic acid present in the reaction mixture, there is a slight decrease in the mycelial weight. This decline may be because of 'autolysis' as the substrate has almost been consumed.

The results of the run with 2% tannic acid concentration as shown in Fig. 4, indicate that the rate of consumption is very fast for the first 20 hours of incubation but afterwards it is very slow throughout the reaction. In this case, again, there is no accumulation of gallic acid initially for approximately 10 hours even though the tannic acid is being consumed. The gallic acid concentration increases after this initial period to reach a maximum value at about 28 hours after the incubation and from then it decreases slowly. The yield coefficient at the maximum point is 0.46.

It is seen from Fig. 4, that there is no lag phase for the growth of the fungus. This may be due to the fact that since the initial inoculum was prepared at the same tannic acid concentration, (i.e. 2% tannic acid) the organisms were already adapted to this concentration, thus requiring no lag period for their growth. The growth rate increases, reaches a maximum value when the time is about 55 hours. After attaining the maximum, there is a slight decline in the mycelial weight which may be, again due to autolysis.

In the case of 5.0% tannic acid concentration, the results of which are shown in Fig. 5 and tabulated in Table V, the rate of utilization of tannic acid is very fast during the initial 12 hours, afterwards the rate slowly decreases. For this case also, there is almost no formation of gallic acid for the first 8 - 12 hours. The rate of formation of the acid increases significantly, only after 12 hours and reaches a maximum when the time of incubation is about 36 hours. Afterwards the concentration of gallic acid decreases slowly to reach more or less a constant value. The yield coefficient for this case was found to be 0.35.

For this case also there is a lag phase of about 12 hours showing apparently no growth of organisms as in the case of 0.5% tannic acid substrate. This may be again due to the fact that the organisms take certain time to adapt themselves to

the new substrate concentration. Mycelium growth starts to increase after the lag phase and reaches a maximum value at about 59 hours. After this point, there is a slight decline in the dry weight of mycelia, as in the case of the two runs with 0.5 and 2.0% tannic acid concentrations.

Comparison of Figures 3, 4 and 5 indicate that the trend of the results is same for all the three concentrations. Table VI summarizes the results for the three concentrations. It gives the values of the lag phase period, yield coefficient, time for maximum product formation, time at which gallic acid accumulation begins and the maximum mycelial weight for the three sets.

Kinetics of Growth:

In order to check whether cube root law or exponential law is followed by the growth of *Aspergillus niger* on tannic acid media, least-square method with 95 percent confidence limits was used to determine the correlation coefficient and the sum of the squares of the errors for the two cases. Also the slope as well as the upper and lower bounds on the slope were calculated in each case.

Table VII indicate the values of correlation coefficient, slope, 95 percent confidence limits and the sum of the squares of

TABLE VI

A COMPARISON OF EXPERIMENTAL RESULTS

Initial Tannic Acid Concentration % →	0.5	2.0	5.0
1. Yield Coefficient	0.20	0.46	0.35
2. Time for Maximum Product Formation hours	24	28	36
3. Approximate Lag Phase Period hours	8	0	8
4. Approximate Time at which Gallic Acid Accumulation starts, hours	8	10	12
5. Maximum Mycelial Weight, mg/50 ml.	123.7	147.1	89.5

the errors for the cube root law, and Table VIII shows the same values for exponential law. Comparison of the two tables indicate that the values of the correlation coefficient, R for cube root case is slightly better than that for the exponential case for all the three substrate concentrations. In addition, the values of sum of squares of errors are less in the case of cube-root law compared to those for the exponential law except for a single discrepancy. This discrepancy exists for the run with 0.5% tannic acid concentration. The reasons for this discrepancy are not known. Based on the results of these two tables, it may be inferred with some reservations, that the growth follows cube root law more closely than the exponential law.

* * *

TABLE VII

GROWTH KINETICS OF A. NIGER

CUBE-ROOT LAW

Tannic Acid Concentration %	Correlation Coefficient R	Slope	95% Confidence Limits on Slope		Intercept	Sum of Squares of Errors
			Upper	Lower		
0.5	0.9872	0.04035	0.04940	0.03131	2.9565	0.03458
2.0	0.9986	0.04442	0.04648	0.04237	2.8225	0.00950
5.0	0.9944	0.03947	0.04529	0.03365	2.2874	0.01804

TABLE VIII

GROWTH KINETICS OF A. NIGER

EXPONENTIAL LAW

Tannic Acid Concentration %	Correlation Coefficient R	Slope	95% Confidence Limits on Slope		Intercept	Sum of Squares of Errors
			Upper	Lower		
0.5	0.97822	0.0281	0.0363	0.0198	3.4211	0.02893
2.0	0.99652	0.0324	0.0349	0.0300	3.2535	0.01352
5.0	0.98877	0.0313	0.0379	0.0247	2.7749	0.02309

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

A. Conclusions:

Based on the results of the present study, the following conclusions can be made:

1. For a 2% tannic acid substrate concentration, a pH of 5.5 and temperature of 30°C appear to be the most favourable conditions for the conversion of tannic acid to gallic acid.

2. A maximum yield of 46% gallic acid is obtained when the substrate was 2% tannic acid solution.

3. The growth of *Aspergillus niger* on tannic acid substrate follows the cube-root law more closely than the exponential law.

B. Recommendations:

The following are some of the suggestions for further work.

A large number of experiments should be performed under various conditions so that a law for the growth rate of the organisms is established more precisely. This data can also be utilized to determine the best operating conditions for the conversion.

The results show that concentrations of 5% or more of tannic acid are toxic to the organisms. This is evident from the fact that the growth is less in the case of 5% concentration as compared to 0.5 and 2.0% cases. Obviously, the quantity of the gallic acid produced will be very low, when low concentrations of tannic acid are used in the hydrolysis reaction. Therefore, attempts are to be made to separate the enzyme'tannase'. Since toxicity does not enter into the picture when tannase is used as the hydrolysing catalyst, higher concentrations of tannic acid can be used for its conversion to gallic acid. This method might prove to be a commercially feasible one to produce gallic acid from tannins. The methods of purification of tannase are reported by N. Hiroshi and N. Mugibayashi (11), H. Yamada et.al. (12) and S. Iibuchi (15).

Some improvement should be made in the method of estimating tannic acid and gallic acid in the culture liquid samples. Some of the recently developed methods are discussed by Iibuchi, et.al. (16), Nishira (17, 18), Kimura, et.al. (19) and Mitjavila (20).

Hiroshi Nishira (21) recommends the addition of 2,4,dinitrophenol to the secondary culture to inhibit the decomposition of gallic acid. This does not cause considerable inhibition of enzymatic hydrolysis of tannin to gallic acid.

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APPENDIX - A

The procedure for the determination of the dry weight of mycelium, and the quantities of tannic and gallic acid in the samples was as given below:

Dry Weight:

The sample was filtered and washed thoroughly with distilled water. The mycelium mat was collected on a pre-weighed filter paper. The filter paper with its contents were transferred to a petri-dish and kept in an oven at 90°C for 24 hours. It was cooled in a desicator and its weight was determined to the fourth decimal place by an analytical balance. From this, the dry weight of the mycelium was calculated.

Determination of Tannic and Gallic Acid:

An accurate colorimetric (based on absorbance) method of estimating gallic acid in the presence of gallotannin as explained by Mitchell (14, [illegible]) was used. It is based on the fact that the presence of a tartrate causes gallic acid or gallotannin to react immediately with ferrous sulphate to form a soluble compound which is fairly stable. This method is capable of detecting 0.0001 gm of gallic acid in 100 cc.

The reagent consists of a solution of 0.1% of ferrous sulphate and 0.5% of Rochelle salt (Sodium Potassium Tartrate).

In estimating gallic acid in the presence of gallotannin, the two substances are first estimated together colorimetrically as described, and the results are expressed in terms of pure gallic acid. An aliquot portion of the solution is then treated with a slight excess of quinnine hydrochloride to precipitate out the tannins completely, leaving the gallic acid in solution The analysis of the filtrate, in the same way as explained earlier, will give the amount of gallic acid originally present in the mixture. The difference of these two gives the amount of tannic acid expressed as gallic acid equivalent. Knowing the colorimetric ratio between pure tannic acid and pure gallic acid, the quantity of tannic acid directly can be determined.

In the precipitation steps the quinine - tannate that is formed will be in colloidal form. In order to facilitate easy filtration, addition of a small quantity of sodium chloride is recomended for coagulating purpose.

Procedure:

2 mls. of freshly prepared color reagent is added to an aqueous solution of gallic acid sample in a 100 ml flask. A violet color developed when the solution was buffered to a pH of 7 by the addition of 10 mls of 10% solution of ammonium

acetate and sufficient distilled water to adjust the final volume of the reaction mixture to 100 mls. The absorbance of the sample were determined at 540 $m\mu$ using spectromic '20'. The absorbance was found maximum at 540 $m\mu$. The concentration of gallic acid employed in the standardization ranged between 0.1 and 1.0 mg per 100 ml. of the solution. The blank contained only the color reagent and the buffer.

For the analysis of mixtures of tannic acid and gallic acid, the same procedure was adopted to determine the two estimations as described earlier. For these cases, 0.5 ml of 10% sodium chloride solution was added to the sample so that the quinine-tannate is coagulated for easy filtration. The standard (blank) also contained the same quantity of sodium chloride in addition to buffer and the color reagent.

<u>Calibration Curve for Gallic Acid:</u>

Sl.No.	Gallic Acid, mg/100 ml. of solution	Absorbance
1.	0.2	0.037
2.	0.3	0.055
3.	0.4	0.070
4.	0.5	0.087
5.	0.6	0.105
6.	0.7	0.120
7.	0.8	0.138
8.	0.9	0.157
9.	1.0	0.170

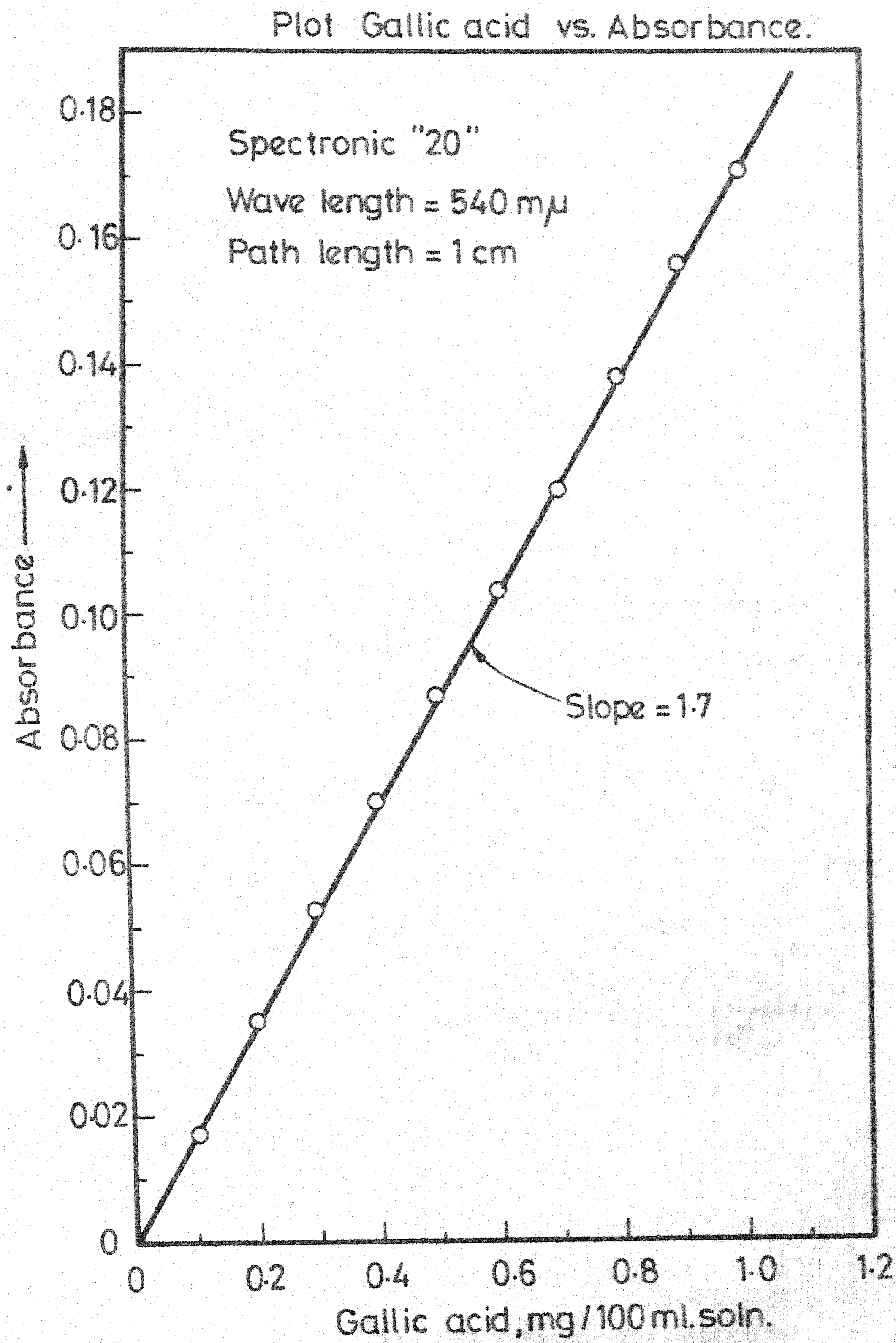

Fig. 6 - Calibration curve Gallic Acid.

In order to determine the colorimetric ratio between tannic acid and gallic acid, an aqueous solution of tannic acid of known concentration was taken in a 100 ml.flask. 2 ml. of color reagent was added and the solution buffered to a pH of 7 by the addition of 10 ml of 10% solution of ammonium acetate. The absorbance of sample was determined at 540 mμ in spectronic 20. Using the calibration curve for gallic acid, Fig. 6, the equivalance of gallic acid was determined.

Tannic acid 1 ml. of 0.1% solution gives absorbance = 0.12.

From Fig. 6, corresponding to this absorbance, amount of gallic acid = 0.7 mg/100 ml of solution.

Tannic acid : Gallic acid

1 : 0.7

1.43: 1

Colorimetric ratio is 1.43 to gallic acid

$$\frac{\text{Tannic acid}}{\text{mg/ml}} = \frac{\text{Colorimetric}}{\text{Ratio}} \times \frac{\text{Tannic Acid equivalent}}{\text{gallic acid mg/ml.}}$$

* * *

APPENDIX - B

THEORETICAL CALCULATIONS

Determination of Yield Coefficient

$$\text{Yield Coefficient} = \frac{\text{Amount of Gallic Acid Formed}}{\text{Amount of Tannic Acid Consumed}}$$

$$= \frac{G_t - G_o}{T_o - T_t}$$

where G_t = Amount of Gallic Acid at time t.

G_o = Amount of Gallic Acid initially.

T_t = Amount of Tannic Acid at time t.

T_o = Amount of Tannic Acid initially.

(a) 0.5% tannic acid

$$\text{Yield Coefficient} = \frac{2.80 - 2.08}{4.17 - 0.57} = \frac{0.72}{3.60} = 0.20$$

(b) 2% tannic acid

$$\text{Yield Coefficient} = \frac{11.50 - 7.0}{13.5 - 3.7} = \frac{4.5}{9.8} = 0.46$$

(c) 5% tannic acid

$$\text{Yield Coefficient} = \frac{20.00 - 13.60}{36.00 - 17.85} = \frac{6.40}{18.25} = 0.35$$

* * *